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Response to: The *GPRC5A* frameshift variant c.183del is not associated with increased breast cancer risk in *BRCA1* mutation carriers

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Dear Editor,

In their letter to the Editor, Klaschik et al. argue that deletion of a large portion of *GPRC5A* gene in human breast cancer cell line MDA-MB-231 has no effect on expression of BRCA1, does not induce apoptosis, and does not sensitize cells to carboplatin. They also use a large data set to demonstrate that germ-line mutations in *GPRC5A* are not more frequent in *BRCA1* mutation carriers compared with the general population. Based on these two lines of evidence, they conclude that the role of GPRC5A in initiation and progression of breast cancer is of minor importance, thus contradicting our earlier suggestions.

Our initial conclusion about mutual effects on gene expression between *GPRC5A* and *BRCA1* was made based on siRNA-mediated knock-down in MDA-MB-231 cells as detected by immunofluorescence and qPCR. This effect was later independently reproduced in HeLa cells using siRNA- and shRNA-mediated knock-down and detected by Western blotting (see Fig. 1A & B). We also noticed that shRNA-mediated depletion of GPRC5A usually produced a milder cellular phenotype compared with siRNA-mediated knock-down (data not shown), which could be explained by adaptations or phenotypic selection taking place over the course of several weeks needed to establish a stable cell line carrying a shRNA in contrast to a transient transfection of siRNA taking only a couple of days. Klaschik et al. used CRISPR/Cas9-mediated *GPRC5A* knock-out (KO) in their experiments. Although this should in theory provide a better experimental model entirely lacking a functional protein, it requires a very long period of cell cloning and selection and increases a potential negative selection if the target gene is essential for cell growth and viability. As a result, the *GPRC5A* knock-out cells generated by Klaschik et al. would be less likely to show some effects visible in quick siRNA-experiments. We would also like to point out that the experimental data of Klaschik et al. appear to be based on a single clone of *GRPC5A* knock-out cell line (and three control clones), which may not be representative of a wider cell population.

Although we do believe that depletion of GPRC5A indeed has a negative effect on BRCA1 (and vice versa), its magnitude varied between different HeLa and MDA-MB-231 cell lines. In addition, one of our shRNAs targeting *GPRC5A* appeared to increase rather than decrease BRCA1 level (lane 2 in Fig. 1B). However, this shRNA also produced a number of unusual phenotypic changes that were not observed with other GPRC5A-targeting reagents, suggesting an off-target effect (data not shown).

Perturbations of GPRC5A and BRCA1 seem to involve also *p53* and *ATR* genes (Fig. 1A), suggesting that the relationship between GPRC5A and BRCA1 may be indirect, possibly mediated by p53.

Klaschik et al. did not observe any significant differences between *GPRC5A* KO and wild-type clones when the level of PCNA was measured by Western blotting and concluded that GPRC5A has no effect on cell proliferation. However, a bulk PCNA protein level cannot reliably reveal small differences between cell populations. Immunocytochemical detection of a PCNA-positive cell population or the number of mitotic figures would be a better marker of proliferation in their case. We used an IncuCyte automatic imaging system (Sartorius) measuring cell confluence every 3 hours as a proxi for cell proliferation and found that suppression of *GPRC5A* inhibits cell proliferation at least in MDA-MB-231 (siRNA-mediated knockdown, Fig. 1C) and HeLa (CRISPR/Cas9-mediated knockout, Fig. 1D) cells by 10% - 15%, while overexpression of GPRC5A using a specific version of activating Cas9 construct seems to improve cell proliferation in HeLa cells (Fig. 1D). In fact, data showing a reduced proliferation of MCF7 and T47D breast cancer cell lines after GPRC5A depletion have been published earlier by others.[1] However, we do not yet understand the mechanistic link between GPRC5A and cell proliferation. Klaschik et al. did not observe an increase in Caspase-3 in *GPRC5A* KO cells on a Western-blot. Neither did we observe any increase in a fraction of annexin V-positive HeLa cells stably expressing GPRC5A-targeting shRNA (data not shown). However, we did see a strong increase of cleaved PARP, another apoptotic marker, in three cell lines (HeLa, MDA-MB-231, MCF10A) after depletion of GPRC5A with siRNA (Fig. 1G) indicating that apoptotic pathway has been activated at least to some extent. In addition, similar to MDA-MB-231 cells shown earlier[2], we found a slightly reduced radiation-induced RAD51 nuclear foci formation in HeLa cells independently depleted for GPRC5A with 2 siRNAs, although the number of foci was much higher compared with depletion of BRCA1 (Fig. 1E). In our hands, HeLa cells stably expressing shRNA targeting GPRC5A were also slightly more sensitive to ionizing radiation (Fig. 1F). This result appears to contradict the carboplatin sensitivity data by Klaschik et al. However, they measured cell viability 48 h after starting carboplatin treatment, which may not be enough time to reveal small differences in sensitivity. We typically perform measurements 3-5 days after beginning a drug treatment. Cell adaptation and clonal selection during KO cell generation could also play a role.

Klaschik et al. did not observe any significant correlation between *GPRC5A* and *BRCA1* germline mutations in an impressive collection of several thousands of breast cancer samples in contrast to our earlier findings. Now we analyzed 118 additional samples with *BRCA1* 5382insC allele and found the *GPRC5A* c.183delG mutation present 3 (2.5%) cases, which was still higher than in *BRCA1* mutation-negative breast cancer patients (8/1578 0.5%; see Sokolenko et al.[2]). We agree that our data are still too small to support the link between GPRC5A and BRCA1-mediated breast cancer risk. We speculate that this over-representation of *GPRC5A* c.183delG variant among *BRCA1* heterozygotes could be limited to certain *BRCA1* mutations (e.g., *BRCA1* 5382insC), possibly due to simultaneous persistence of two founder alleles in a given population.

Based on their genetic data and a study by Jorissen and colleagues,[3] Klaschik et al. conclude that *GPRC5A* is of minor significance for breast cancer. In contrast, our new data suggest that breast cancer is characterized by significant changes in GPRC5A expression level. First, a higher expression of GPRC5A in a panel of 3951 microarray samples publicly available in the Kaplan-Meier Plotter resource (kmplot.com)[4] is associated with a significantly lower relapse-free survival (Supporting Information Fig. S1A). Second, we detected a significantly higher level of GPRC5A protein in malignant mammary tumors compared with benign cases when we stained a commercial tissue microarray purchased from US Biomax, Inc., using a GPRC5A antibody extensively characterized within the Human Protein Atlas project (Supporting Information Fig. S1B). Third, using this tissue microarray, we found a direct correlation between GPRC5A and the estrogen receptor status within all breast cancers, on the one hand, and between GPRC5A and a proliferation marker Ki67 within ER-positive, but not ER-negative invasive ductal carcinomas, on the other hand (Supporting Information Fig. S1C and D). This correlation was even stronger at the mRNA level (Supporting Information Fig. S1E and F). A connection between GPRC5A and estrogen signaling is further supported by induction of GPRC5A protein expression in MCF7 cells treated with beta-estradiol (Supporting Information Fig. 1G). Taken together, these data suggest a role for GPRC5A in breast cancer progression possibly associated with estrogen receptor signaling, while its role in BRCA1-mediated breast cancer risk remains uncertain.

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FIGURE LEGEND

Figure 1. GPRC5A affects cell proliferation. A, Western blot showing HeLa cells with (+) or without (-) shRNA targeting *GPRC5A* 60 hours after transfection with siRNAs listed above and probed with antibodies against proteins listed on the left. **B**, Western blot showing effect on BRCA1 protein expression in HeLa cells expression different shRNAs against *GPRC5A* (shGPRC5A) or non-targeting control (shControl). **C**, Growth curve of MDA-MB-231 cells plated in IncuCyte imager at the same density 48 h after a transient transfection with listed siRNAs. A total culture confluency has been measured automatically every 3 h. **D**, Growth curve of HeLa cells carrying either CRISPR/Cas9 construct targeting *GPRC5A* (KO) or an activating CRISPR/Cas9a construct upregulating GPRC5A level (ACT), or non-targeting CRISPR/Cas9 construct (Control). Cell confluence has been measured using IncuCyte imager. **E**, HeLa cells transfected with siRNAs targeting *GPRC5A* produce slightly fewer radiation-induced nuclear RAD51 foci. The assay was performed exactly as described in Sokolenko et al.[2], and foci quantification and curve fitting were performed automatically. **F**, HeLa cells stably expressing shRNA targeting *GPRC5A* (shGPRC5A) are more sensitive to gamma-irradiation than control cells (shControl). Cell proliferation was measured 3 days after irradiation using an Alamar Blue metabolic assay. Cells treated (RA+) or not treated (RA-) with 100 nM all-trans retinoic acid are shown. G, Western blot showing the effect of GPRC5A depletion (siGPRC5A) and retinoic acid treatment (atRA) on PARP cleavage in 3 different cell lines listed above.